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Intercellular Communication via Connexin43 Gap Junctions Is Required for Ovarian Folliculogenesis in the Mouse

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The ovarian follicle in mammals is a functional syncytium, with the oocyte being coupled with the surrounding cumulus granulosa cells, and the cumulus cells being coupled with each other and with the mural granulosa cells, via gap junctions. The gap junctions coupling granulosa cells in mature follicles contain several different connexins (gap junction channel proteins), including connexins 32, 43, and 45. Connexin43 immunoreactivity can be detected from the onset of folliculogenesis just after birth and persists through ovulation. In order to assess the importance of connexin43 gap junctions for postnatal folliculogenesis, we grafted ovaries from late gestation mouse fetuses or newborn pups lacking connexin43 (*Gja1*$^{-}$/*Gja1*$^{-}$) into the kidney capsules of adult females and allowed them to develop for up to 3 weeks (this was necessitated by the neonatal lethality caused by the mutation). By the end of the graft period, tertiary (antral) follicles had developed in grafted normal (wild-type or heterozygote) ovaries. Most follicles in *Gja1*$^{-}$/*Gja1*$^{-}$ ovaries, however, failed to become multilaminar, with the severity of the effect depending on strain background. Dye transfer experiments indicated that intercellular coupling between granulosa cells is reduced, but not abolished, in the absence of connexin43, consistent with the presence of additional connexins. These results suggest that coupling between granulosa cells mediated specifically by connexin43 channels is required for continued follicular growth. Measurements of oocyte diameters revealed that oocyte growth in mutant follicles is retarded, but not arrested, despite the arrest of folliculogenesis. The mutant follicles are morphologically abnormal: the zona pellucida is poorly developed, the cytoplasm of both granulosa cells and oocytes is vacuolated, and cortical granules are absent from the oocytes. Correspondingly, the mutant oocytes obtained from 3-week grafts failed to undergo meiotic maturation and could not be fertilized, although half of the wild-type oocytes from 3-week grafted ovaries could be fertilized. We conclude that connexin43-containing gap junction channels are required for expansion of the granulosa cell population during the early stages of follicular development and that failure of the granulosa cell layers to develop properly has severe consequences for the oocyte.

Key Words: **oogenesis; folliculogenesis; gap junctions; connexin43; Cx43; gene targeting; ovary graft; intercellular communication.**

INTRODUCTION

Gap junctions occur at sites of close cell apposition; they are arrays of intercellular membrane channels that allow inorganic ions, second messengers, and small metabolites (less than about 1000 Da) to pass from cell to cell (reviewed by Bruzzone *et al.*, 1996a,b). The fundamental unit of the gap junction is the connexon, a cylindrical organelle that forms a hemichannel in the membrane. It is the end-to-end docking of connexons from two adjacent cells that creates the intercellular channel. Each connexon is a hexamer of protein subunits called connexins (Unger *et al.*, 1999). The connexins are a family of at least 15 proteins, each the product of a distinct gene (Bruzzone *et al.*, 1996a,b; Dahl *et al.*, 1996; Condorelli *et al.*, 1998; Manthey *et al.*, 1999;

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Al-Ubaidi *et al.*, 2000). Connexins differ greatly in size, with connexin26 (Cx26 or β2 connexin) being the smallest of the known rodent connexins at 26 kDa and connexin57 (Cx57 or α10 connexin) being the largest at 57 kDa. All have four membrane-spanning domains, two extracellular loops, a cytoplasmic loop, and cytoplasmic N- and C-termini. Sequence similarity among the family members is concentrated in the transmembrane domains and extracellular loops, whereas most of the sequence and length variation resides in the cytoplasmic loops and C-terminal tails. This diversity is assumed to account for most of the distinct biophysical, permeability, and regulatory properties that are exhibited by gap junction channels composed of different connexins (Bruzzone *et al.*, 1996a,b). Each connexin has a characteristic tissue distribution, although most organs (and even some individual cell types) express more than one (Bruzzone *et al.*, 1996b). These facts support the hypothesis that channels formed of different connexins play distinctive roles. The potential diversity of channel types generated by the diversity of connexins is further magnified by the existence of heterotypic channels, formed by pairing of two homomeric connexons, each composed of a different connexin, as well as heteromeric channels, formed from connexons composed of a mixture of two (or more) connexins (Bruzzone *et al.*, 1996a,b; He *et al.*, 1999). Given the different properties of the various connexins this can and does result in complex possibilities with regard to the selectivity and regulation of intercellular coupling (He *et al.*, 1999). On the other hand, the biological significance of connexin diversity is still not fully understood and the extent of functional redundancy among the members of this gene family is just beginning to be explored (Houghton *et al.*, 1999). Definitive evidence that gap-junctional intercellular coupling (GJIC) plays essential roles in organogenesis has come from connexin gene-targeting experiments that have generated a variety of developmental and physiological abnormalities; in addition, several human congenital abnormalities are known to be associated with connexin mutations (reviewed by White and Paul, 1999; Krutovskikh and Yamasaki, 2000).

The ovarian follicle provides a clear example of the importance of GJIC in a developmental process. The follicle is a functional syncytium with the oocyte coupled with the granulosa cells and the granulosa cells with each other via gap junctions (reviewed by Eppig, 1991; Eppig *et al.*, 1996). According to morphological evidence, this coupling begins as primordial follicles form (around the time of birth in the mouse) and expands as folliculogenesis proceeds through primary, secondary, and tertiary (antral) follicle stages (Mitchell and Burghardt, 1986). Amino acids, glucose metabolites, and nucleotides are among the molecules known to be transferred to the growing oocyte via gap junctions (Eppig, 1991). In addition, signals which regulate meiotic maturation of the fully grown oocyte pass through the oocyte–granulosa cell gap junctions (Coskun and Lin, 1994; Granot and Dekel, 1994; Downs, 1995; Byskov *et al.*, 1997). These communication pathways involve multiple connexins. In rodents, Cx32 (β1 connexin) and Cx43 (α1 connexin) have been identified in gap junctions joining the cumulus granulosa cells of fully grown follicles (Valdimarsson *et al.*, 1993; Li and Mather, 1997). In the case of Cx43 this expression begins as early as postnatal day 1, when the first primordial follicles are forming (Juneja *et al.*, 1999). Cx45 (α7 connexin) is also present in developing follicles, colocalizing with Cx43 in some granulosa cell gap junctions (Okuma *et al.*, 1996; Alcoléa *et al.*, 1999). A fourth connexin, Cx37 (α4 connexin), is present from the primary follicle stage at the interface between oocytes and cumulus granulosa cells (Simon *et al.*, 1997) and is expressed within the granulosa layers in later stages (Wright *et al.*, 2000). A fifth connexin likely to play a role in oogenesis is Cx57 (α10 connexin; Manthey *et al.*, 1999). This newly cloned mouse connexin, the gene for which is transcribed in several adult organs, including the ovary, is a homolog of Cx60, which is expressed in cumulus (but not mural) granulosa cells as well as theca cells in pig ovarian follicles (Itahana *et al.*, 1998).

Our interest has been in the role of Cx43 in folliculogenesis. Disruption of the gene (*Gja1*) encoding this connexin results in neonatal lethality because of a severe heart abnormality (Reaume *et al.*, 1995); in addition, the homozygous mutant fetuses have very few germ cells (Juneja *et al.*, 1999). This is evident from as early as 11.5 dpc, when the first primordial germ cells (PGCs) have taken up residence in the genital ridges, suggesting that the deficiency could arise during PGC migration. Despite this, roughly 10% of germ cells remain in the gonads of Cx43-deficient neonates, raising the possibility that these mice might be fertile if not for the lethality of the heart defect. We tested this hypothesis by culturing neonatal ovaries *in vitro*, revealing that postnatal folliculogenesis, specifically development of the granulosa cell layers, is impaired in the absence of Cx43 (Juneja *et al.*, 1999). However, because of limitations of the culture system, we could follow the progress of folliculogenesis in the mutant follicles for only 8 days and thus were unable to determine whether the mutation causes complete arrest or merely a retardation of folliculogenesis. In the present study, we used a kidney graft procedure to follow folliculogenesis in $Gja1^{-}/Gja1^{-}$ ovaries for up to 3 weeks after birth. The results demonstrate that, in the absence of Cx43, folliculogenesis arrests before the follicles become multilaminar, with severe consequences for the developing oocytes.

MATERIALS AND METHODS

Mice

All mice used as graft recipients were 18- to 20-g $Prkdc^{scid}/Prkdc^{scid}$ females (C.B-17/IcrHsd-*scid* purchased from Harlan Sprague–Dawley, Indianapolis, IN or obtained from a research colony maintained at The Jackson Laboratory). Offspring lacking Cx43 were obtained by mating heterozygotes ($Gja1^{+}/Gja1^{-}$) maintained on either the C57BL/6J or the CD1 genetic background. The

ovary donors consisted of neonates or late gestation fetuses, the latter delivered by cesarean section on day 17.5 of gestation from dams killed by CO_2 anesthesia followed by cervical dislocation. Offspring were killed by decapitation before removal of the ovaries. The genotypes of the ovary donors were determined by PCR applied to proteinase K-digested tail snips using three sets of primers. For the *Gja1* wild-type allele the primers were 5′-CCCCAC-TCTCACCTATGTCTCC-3′ and 5′-ACTTTTGCCGCCTAGCT-ATCCC-3′ and they generated a 519-bp amplicon. For the *Neo* (disrupted) allele the primers were 5′-CTTGGGTGGAGAG-GCTATTC-3′ and 5′-AGGTGAGATGACAGGAGATC-3′, generating a 280-bp amplicon. These two PCRs were run in separate tubes. A third set of primers was used as a positive control for each PCR; these primers were designed to amplify a 206-bp segment of the T cell receptor gene. The sequences were 5′-CAAATGTTG-CTTGTCTGGTG-3′ and 5′-GTCAGTCGAGTGCACAGTTT-3′. Each PCR mixture contained either the *Neo* or the *Gja1* primers (0.2 μM each) plus each of the positive control primers (0.2 μM) in 20 mM Tris–HCl, pH 8.4, containing 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 50 mM KCl, and 0.125 units of Platinum *Taq* polymerase (GIBCO BRL, from Life Technologies, Burlington, Ontario) in a 10-μl volume. Cycling conditions were 3-min soak at 94°C, 94°C for 20 s, 64°C for 30 s (with the temperature of this step dropping 0.5°C per cycle to eventually reach 58°C), followed by 72°C for 35 s. The last (40th) cycle was followed by a 7-min soak at 72°C.

Grafting of Ovaries

Ovaries were placed in 3.0 ml of Waymouth medium (GIBCO BRL) containing 10% FBS and the ovarian bursa was removed. The ovaries were then transferred to Millicell culture plate inserts with Isopore polycarbonate membrane, 3.0-μm pore size (Millipore), in a drop of the same medium. Each membrane was in turn placed over 1.5 ml of medium in a well of a six-well tissue culture plate (Corning Costar, Corning, New York) and the ovaries were incubated at 37°C. Once the genotype of each donor had been determined, both of its ovaries were grafted into the right kidney capsule of an ovariectomized $Prkdc^{scid}/Prkdc^{scid}$ mouse. The delay between removal of the ovaries from the donor fetus/neonate and grafting into the adult host was no more than 24 h.

Host mice were anesthetized with either of two agents. For experiments at The Jackson Laboratory, Avertin was used: 0.015 ml of 2.5% 2,2,2-tribromoethanol (Aldrich Chemical Co., Inc., Milwaukee, WI) per gram body weight. For experiments at The University of Western Ontario, ketamine/xylazine was used: 0.04 ml per 10 g body weight of a solution containing 5% ketamine–HCl (Ketaset; Ayerst Veterinary Laboratories, Guelph, Ontario) and 0.2% xylazine (Rompun; Bayer, Inc., Etobicoke, Ontario). Ovariectomy was performed by making a small incision on the dorsolateral surface of the mouse, just caudal to the last rib on the right side. The ovarian artery was tied off with a ligature and the ovary was removed. The kidney was brought to the surface of the wound and a small hole was made in the bursa. The ovaries were inserted through the hole under the bursa and the peritoneal cavity closed with three sutures. The skin was closed with two wound clips. A similar incision was made on the left side of the animal and the left ovary was removed in the manner described above. Buprenorphine (0.05 μg Buprenex per gram body weight; from Reckitt and Coleman, Hull, UK) was administered as an analgesic and the mouse was placed on a 37°C circulating water blanket to recover. Once alert, the mouse was returned to its cage and monitored daily. Wound clips were removed after 14 days.

Grafts were removed after 1–3 weeks. The mouse was killed by CO_2 anesthesia followed by cervical dislocation. The graft site was exposed and the grafted ovaries, with the attached kidney capsule, were removed. The graft was placed in 3 ml of PBS and the ovaries were separated from adherent kidney tissue with a pair of 30-gauge needles.

Microscopy

Ovaries (C57BL/6J and CD1 backgrounds) were fixed in Bouin's fixative for 2 h, embedded in paraffin, and sectioned at a thickness of 5 μm. Sections were stained with hematoxylin and eosin. Oocyte diameter measurements were obtained from images captured with a Leica Orthoplan microscope and using Northern Eclipse imaging software (version 5.0 from Empix Imaging, Inc., Mississauga, Ontario). Care was taken to ensure that only oocytes in which the nucleus was clearly visible were measured. For electron microscopy, ovaries were fixed in 2.5% glutaraldehyde in 0.1 M phosphate buffer, embedded in Epon–Araldite, and sectioned at a thickness of 80 nm. They were stained with 2% uranyl acetate and Reynold's lead citrate and sections were viewed with a JEOL 100CX electron microscope at 80 kV.

In Vitro Maturation and Fertilization of Oocytes

Grafted ovaries (C57BL/6J background) were removed from the kidney capsules after 20–22 days. Oocyte–granulosa cell complexes were liberated from follicles and matured for 16 h in Waymouth medium containing 5% FBS (Eppig *et al.*, 1996; Sztein *et al.*, 2000). At this time the oocytes were scored for the presence or absence of a germinal vesicle and polar body. All of the oocytes were then incubated with a freshly prepared epididymal sperm suspension in MEM (GIBCO BRL) containing 3 mg/ml BSA, for 4 h. The sperm were washed off and the oocytes incubated overnight in the same medium. The percentage cleavage was determined at this time. The germinal vesicle status of inseminated oocytes was examined by staining with Hoechst 33258 (Mattson and Albertini, 1990).

Dye Transfer Assay by Preloading

Grafted ovaries (C57BL/6J and CD1 backgrounds) were removed from the kidney capsules after 20 days. Small preantral follicles were dissected out in Waymouth medium containing 10% FBS, taking care to ensure that the follicles selected from wild-type ovaries were comparable in size with those from null mutant ovaries. They were washed through two dishes of the same medium and then placed in 1 ml of fresh medium and incubated at 37°C in an atmosphere of 5% CO_2/5% O_2/90% N_2 for 2 days to allow some of the granulosa cells to settle on the dish. At the same time, ovaries were removed directly from a wild-type female mouse and a culture of granulosa cells was prepared in the same way. These latter cells were preloaded with calcein-AM (a membrane-permeant molecule that gives rise to the membrane-impermeant green fluorescent dye, calcein, once inside the cell) and diI (a lipophilic, fluorescent dye that stains membranes red) as described by Goldberg *et al.* (1995). The cells were then treated with trypsin/EDTA solution (GIBCO BRL) with 200 mg/L DNase for 1–2 min, rinsed, and pipetted onto the unlabeled granulosa cells from the grafted ovaries. The dishes were then incubated for a further 2 h to allow the prelabeled cells to settle out and establish gap junctions with the unlabeled cells. The order of calcein transfer

from preloaded to unlabeled cells (e.g., one cell removed from the preloaded cell, two cells removed, etc.) was taken as an index of the strength of intercellular coupling.

Statistical Analysis

Statistical analysis was performed using Sigma Stat (Jandel Scientific). Comparisons for statistical significance between the experimental and the control were made using a standard *t* test or a Mann–Whitney rank sum test in instances in which the test for normality failed. All tests were performed using a confidence interval of 95%.

RESULTS

Ovarian folliculogenesis in the mouse is a postnatal process. The neonatal lethality of the homozygous *Gja1* null mutation (Reaume *et al.*, 1995) necessitated the grafting of ovaries from late gestation fetuses or neonates into immunocompromised adult hosts so that follicular development could be studied. The ovaries were removed from the host mice; this results in elevation of circulating gonadotropin levels, mimicking the situation in neonatal and juvenile females (Halpin *et al.*, 1986). Figure 1 illustrates the outcome of such an experiment using ovaries from the C57BL/6J strain. After 3 weeks of development in kidney grafts, a range of follicle stages from primordial through primary, secondary (having more than one complete layer of granulosa cells), and tertiary (having an antral cavity) could be seen in wild-type ovaries (Figs. 1A and 1B). These follicles are morphologically normal and previous work has shown that oocytes from antral follicles grown in the kidney capsule can be fertilized *in vitro*, giving rise to normal offspring (Eppig and Wigglesworth, 2000). In contrast, folliculogenesis in grafted ovaries from littermates homozygous for the *Gja1* null mutation did not proceed beyond the unilaminar stage during the same length of time, although the mutant follicles (which were >10-fold fewer in number) had the appearance of normal primary follicles (Figs. 1C and 1D). This finding was consistent among the more than 50 mutant follicles that were examined after 3 weeks of postnatal development. When the time course of folliculogenesis was followed by recovering grafts after 1, 2, and 3 weeks it was apparent that the mutation had not simply caused a delay in the onset of folliculogenesis, but rather that the mutant granulosa cells, once having formed a single cuboidal layer around each oocyte, had not increased in number thereafter (Fig. 2). It appears that granulosa cells in primary follicles lacking Cx43 are unable to proliferate.

The fertilizability of oocytes developing in C57BL/6 grafted ovaries was tested. Ovaries were removed from host kidneys after 20 days and the oocytes liberated from them were matured *in vitro* and incubated with sperm. In three experiments, a total of 31 oocytes from wild-type ovaries and 9 oocytes from mutant ovaries were tested. Oocytes were scored as having matured if the germinal vesicle had broken down and a polar body was visible and as having been successfully fertilized if they subsequently cleaved (Figs. 3A and 3B). Whereas 17 (53%) of wild-type oocytes underwent maturation and 14 of these (82%) cleaved after insemination, none of the mutant oocytes matured or cleaved. Hoechst staining revealed that mutant oocytes still contained intact germinal vesicles (Figs. 3C and 3D). In addition, mutant oocytes appeared to bind sperm in greatly increased numbers in comparison with wild-type oocytes (not illustrated).

The health of oocytes developing in mutant follicles was further assessed by electron microscopy, revealing several morphological abnormalities (Fig. 4). Most obviously, the zona pellucida of mutant oocytes is thinner than that of wild-type oocytes (this made them difficult to handle), and the mutant oocytes themselves are highly vacuolated. Fibrous lattices, a common feature of rodent oocytes (abundantly visible in Fig. 4A), are missing from mutant oocytes. Likewise, the mutant oocytes lack cortical granules. Since cortical granule exocytosis is required for zona hardening and the block to polyspermy (reviewed by Wassarman, 1987), their absence could explain the increase in sperm binding to the mutant oocytes.

Despite the arrest of folliculogenesis and associated oocyte abnormalities in the mutant ovaries, it was clear from the histological analysis (Fig. 2) that the mutant oocytes, like the wild-types, grow continuously during development in the kidney graft. This was quantified by measuring oocyte diameters. At the time of birth there is no difference in diameter between mutant and wild-type oocytes (Fig. 5). After birth, however, the mutant oocytes grow more slowly than their wild-type counterparts. The data for wild-type oocytes in Fig. 5 were obtained by combining measurements from primary, secondary, and tertiary follicles; the same conclusion was reached when the data were separated by individual follicle classes (not shown).

Mouse mutant phenotypes often vary with the strain background, and these differences can provide insight into the existence of other factors affecting the process under study (Threadgill *et al.*, 1995). We therefore examined the effect on folliculogenesis of breeding the *Gja1* null mutation into the CD1 background for at least eight generations. As shown in Fig. 6, the result was slightly different from that described above: although folliculogenesis is disrupted in $Gja1^{-}/Gja1^{-}$ ovaries on the CD1 background, follicles do succeed in developing at least a partial second layer of granulosa cells, although only a very few become truly multilaminar. Figure 7 compares the distribution of follicle types in CD1 wild-type and mutant ovaries after 3 weeks in kidney grafts. Whereas grafted wild-type ovaries exhibited all stages of folliculogenesis, only one early antral stage was seen among the 63 null mutant follicles examined. Most of the mutant follicles reached only the primary or early secondary follicle stage. As on the C57BL/6J background, oocyte growth was retarded in mutant CD1 ovaries with wild-type and homozygous mutant oocytes reaching mean diameters of 75.1 ± 14.1 and 53.9 ± 9.9 μm, respectively,

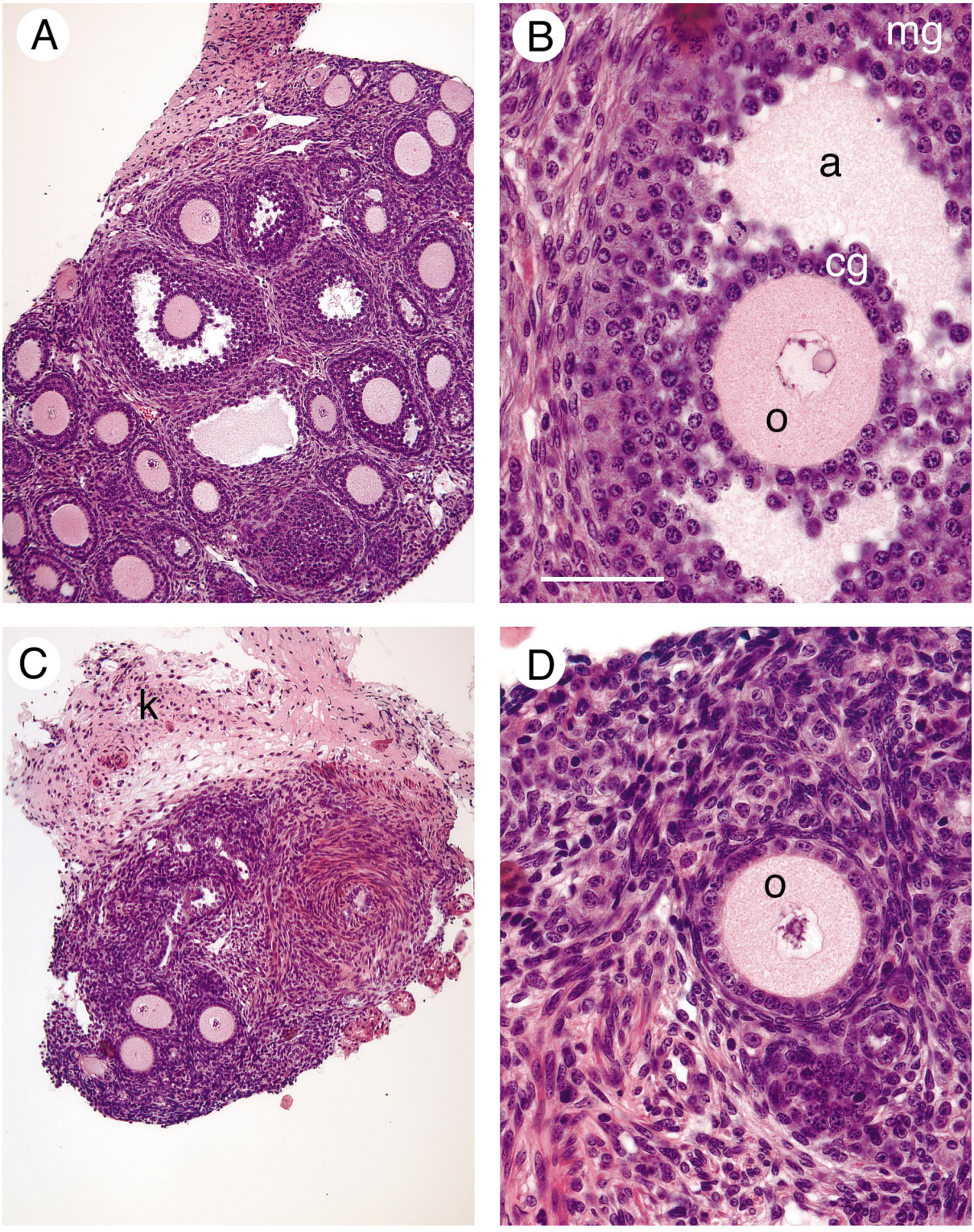

FIG. 1. Folliculogenesis arrests in the primary (unilaminar) follicle stage in the absence of Cx43. Neonatal ovaries (C57BL/6 strain) were grafted into the kidneys of adult *Prkdc*^{scid}/*Prkdc*^{scid} females for 3 weeks. (A, B) Low- and high-magnification images of a wild-type (*Gja1*$^{+}$/*Gja1*$^{+}$) ovary; (C, D) corresponding images of a null mutant (*Gja1*$^{-}$/*Gja1*$^{-}$) ovary. Note the paucity of follicles in the null mutant ovary (Juneja *et al.*, 1999). mg, mural granulosa; cg, cumulus granulosa; a, antrum; o, oocyte; k, kidney tissue. Scale bar, 50 μm.

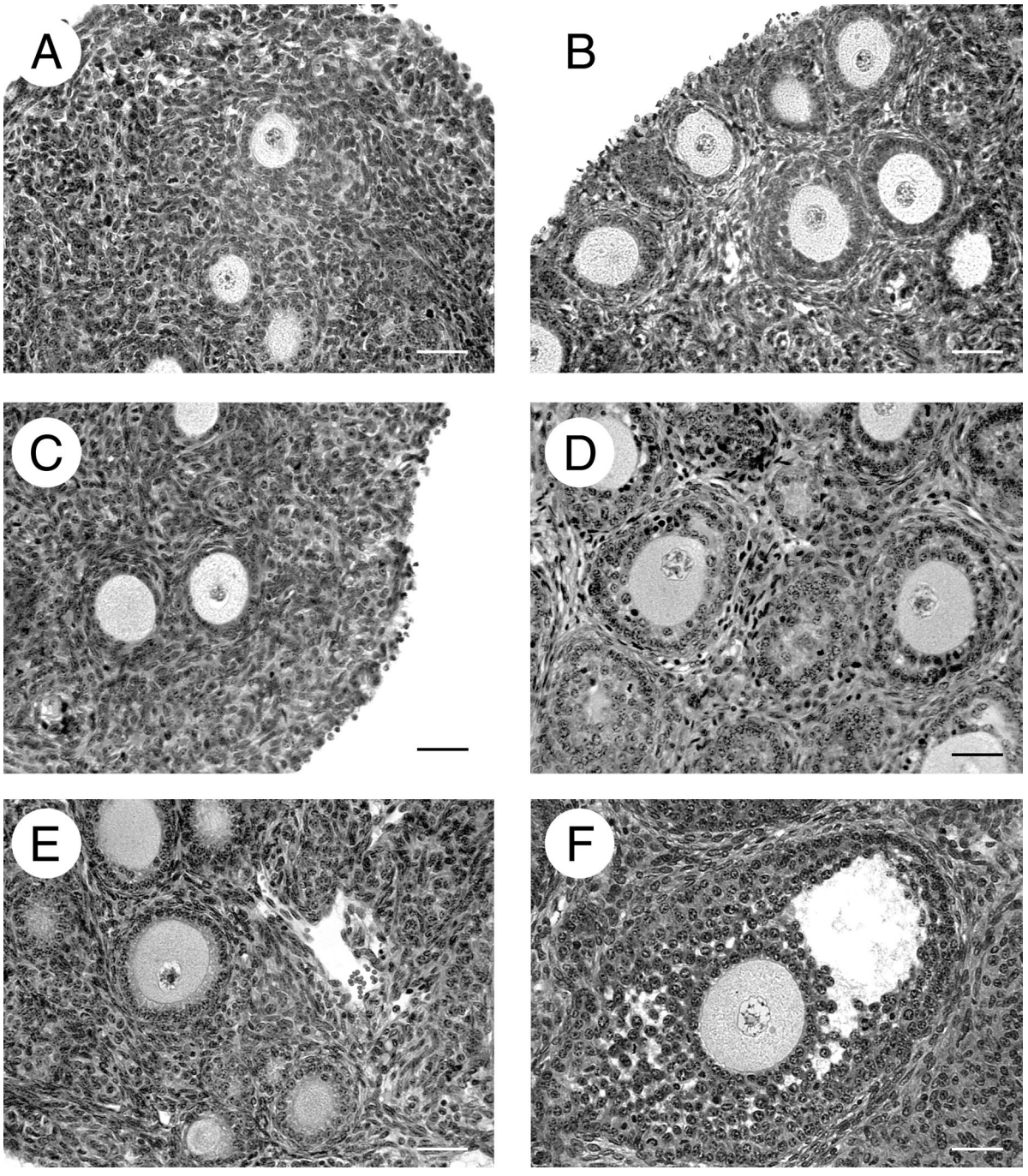

FIG. 2. The onset of folliculogenesis is not delayed by the absence of Cx43. Ovaries (C57BL/6 strain) were recovered after 1 (A, B), 2 (C, D), or 3 weeks (E, F) of postnatal development in kidney grafts. Folliculogenesis began during the first week of postnatal development in both mutant (A, C, E) and wild-type (B, D, F) ovaries. Scale bars, 30 μm.

after 3 weeks of development (difference significant according to Student's t test, $P < 0.0001$).

Finally, a dye coupling assay was used to determine the extent to which intercellular coupling between granulosa cells had been reduced by the removal of Cx43. Mutant and wild-type follicles were isolated from 3-week grafted ovaries of the BL/6 strain and cultured for 2 days to allow the granulosa cells to form monolayers. Granulosa cells from

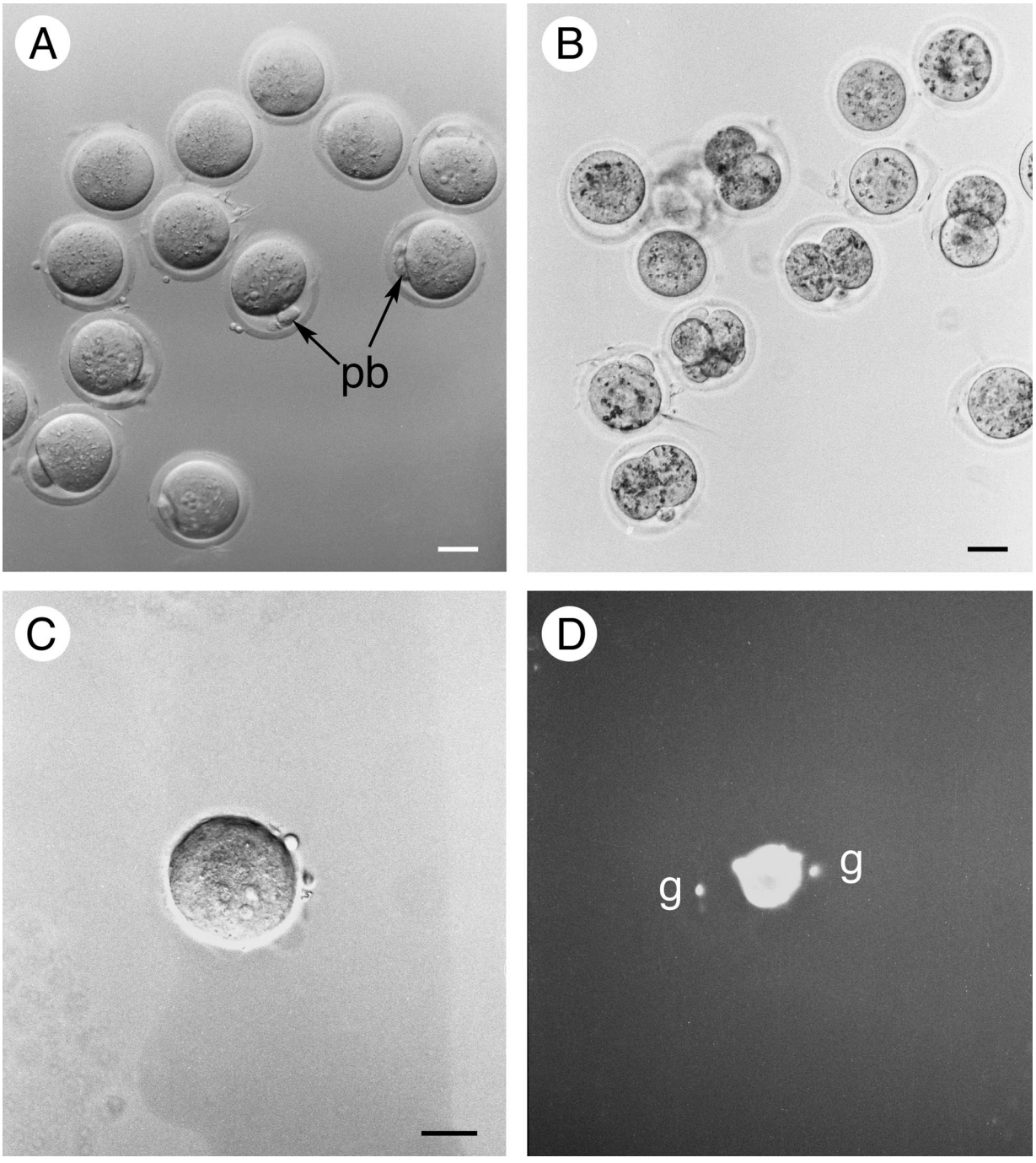

FIG. 3. Oocytes recovered from follicles lacking Cx43 could not be fertilized. After 16 h of culture, 17 of 31 wild-type oocytes had matured (A) as determined by the extrusion of the first polar body (pb). After incubation with sperm, 14 of those oocytes cleaved to the two-cell stage (B). None of the 9 null mutant oocytes tested underwent maturation as indicated by the absence of a polar body (C), nor did any of them cleave after addition of sperm. Hoechst staining (D) revealed that the mutant oocytes had not undergone germinal vesicle breakdown (g, adhering granulosa cells). Scale bars in A and B, 50 μm, in C, 25 μm.

ungrafted wild-type follicles, preloaded with diI and calcein, were then seeded onto the monolayers and allowed time to make gap junctions with the unlabeled cells. Calcein transfer from the preloaded granulosa cells to unlabeled granulosa cells and thence to their neighbors was scored using the red diI label to identify donor cells. Representative dye

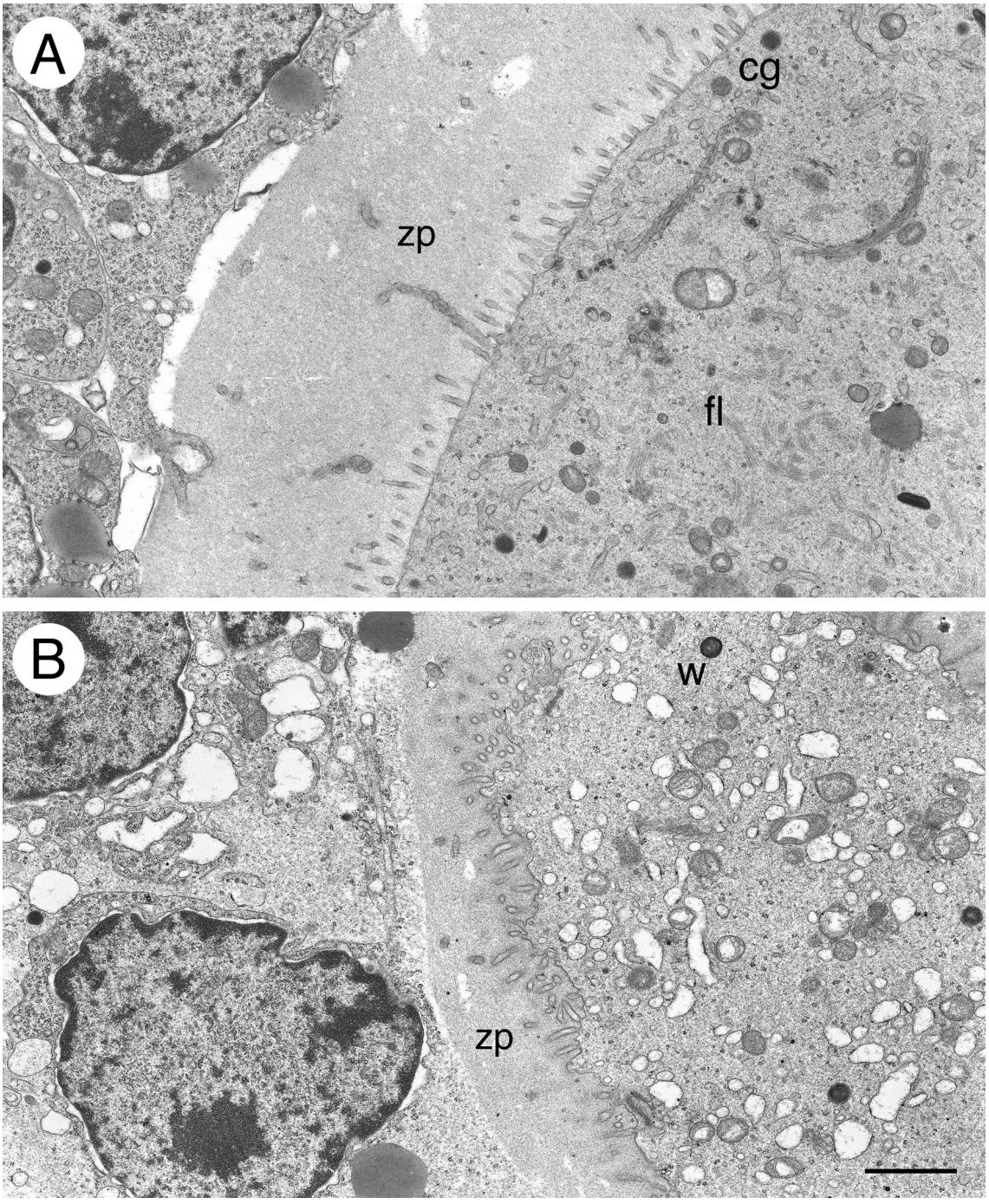

FIG. 4. Oocytes developing in follicles lacking Cx43 are morphologically abnormal. Null mutant oocytes, grown in kidney grafts for 14 days (B), are highly vacuolated and noticeably deficient in fibrous lattices (fl) and cortical granules (cg), structures that are prevalent in wild-type oocytes (A). In addition, the mutant oocytes are highly vacuolated, have an uneven surface, and are surrounded by a thin zona pellucida (zp). Dark whorled structures (w) are common in the mutant oocytes, but were not observed in wild-type oocytes. Scale bar, 1 μm.

spreads are shown in Fig. 8. In both wild-type and null mutant granulosa cells, calcein (green) was passed from preloaded cells (yellow, when diI and calcein images are superimposed) to recipient cells and then on to neighboring cells, indicating the existence of functional gap junction channels. When the extent of dye transfer was tabulated

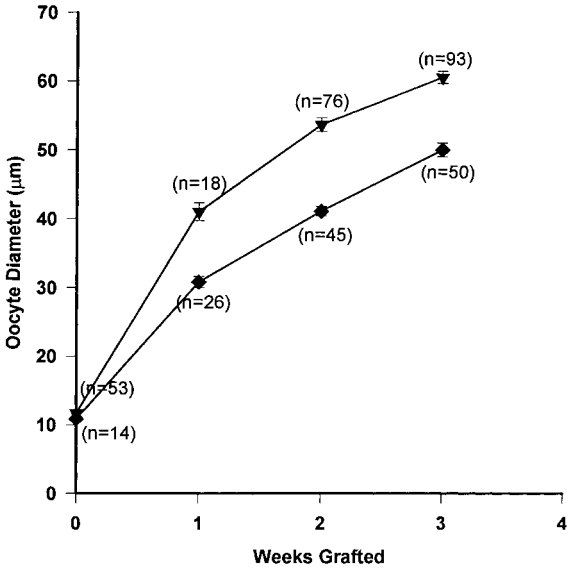

FIG. 5. Oocyte growth is retarded in follicles lacking Cx43. Mean oocyte diameter in null mutant follicles was significantly different from that in wild-type follicles from the first week of postnatal development onward. Error bars represent standard error of the mean; numbers in parentheses indicate the number of oocytes measured. ▼, *Gja1*$^{+}$/*Gja1*$^{+}$; ◆, *Gja1*$^{-}$/*Gja1*$^{-}$.

(taking care to select unambiguous dye spreads in which only one preloaded cell had settled on a colony of unlabeled cells) it was found that wild-type cells passed dye, on average, to neighbors up to 2.14 ± 0.26 cells away from the donor (n = 7 dye spreads), whereas mutant cells passed dye to neighbors up to 1.25 ± 0.16 cells away (n = 8; difference significant according to Student's t test, P = 0.01). These results indicate that gap-junctional coupling between granulosa cells is reduced, but not abolished, in the absence of Cx43. Mutant granulosa cells on the CD1 background also showed residual dye coupling when seeded with preloaded wild type cells from the same background (results not shown).

DISCUSSION

Our results demonstrate that gap-junctional coupling provided by Cx43 channels is necessary for ovarian folliculogenesis. In the absence of this connexin, folliculogenesis does not proceed beyond the primary or early secondary follicle stage (depending on strain background) and the oocytes developing in the mutant follicles are defective. The kidney graft procedure, necessitated by the neonatal lethality of the homozygous *Gja1* null mutation, allowed us to conclude that the developmental lesion is intrinsic to the mutant ovaries and not the result of systemic physiological defects. Efforts are being made to create *Gja1*$^{-}$/*Gja1*$^{-}$ mice using an inducible targeting strategy so that the gene deletion can be delayed until after birth, making it possible to study the postnatal development of other organ systems in the absence of Cx43 (M. Theis and K. Willecke, personal communication). If the deletion occurs in the ovary, our evidence predicts that such females will be sterile.

Within the mouse ovarian follicle Cx43 is abundantly expressed in the cumulus and mural granulosa cells, but its presence at the cumulus-oocyte interface is in doubt despite the detection of Cx43 mRNA in denuded oocytes (Valdimarsson *et al.*, 1993; Simon *et al.*, 1997). Cx43 can be detected in somatic cells of neonatal ovaries at the onset of folliculogenesis (Juneja *et al.*, 1999). Correspondingly, the most obvi-

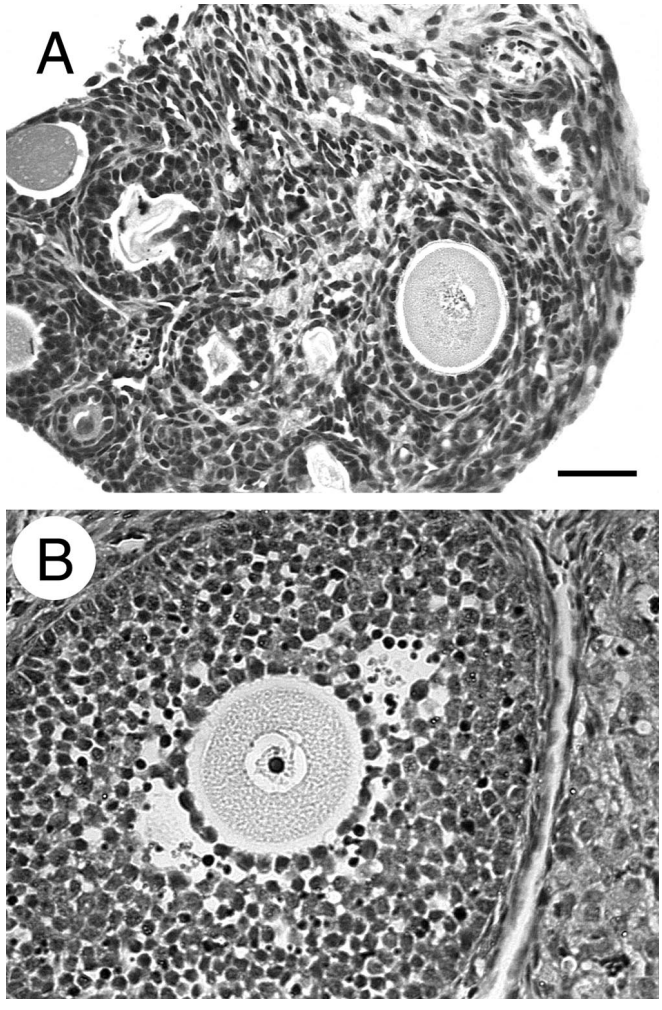

FIG. 6. The extent of follicular development in ovaries lacking Cx43 depends on the strain background. By the third week of postnatal development in kidney grafts, folliculogenesis in CD1 null mutant ovaries was proceeding to the early secondary follicle stage (A), in contrast to the primary follicle arrest seen in mutant ovaries on the C57BL/6 background. Follicles in CD1 wild-type ovaries reached early antral stages in the same time period (B). Scale bar, 30 μm.

ous defect in ovaries lacking Cx43 was the failure of granulosa cells to increase in number to produce multilaminar follicles. The simplest explanation for this phenotype is that granulosa cells require gap-junctional intercellular coupling via channels containing Cx43 to support their continued proliferation. This would be the case, for example, if the sharing of intracellular second messengers generated by the binding of hormones or paracrine factors to receptors on granulosa cells were essential in order for those cells to divide. The most important hormone for early stages of folliculogenesis is FSH, secreted by the pituitary. FSH signals through a G-protein-coupled, seven-transmembrane-domain receptor that is expressed on granulosa cells of the mouse ovary from postnatal day 3 onward (reviewed by Simoni *et al.*, 1997). Binding of FSH to its receptor causes an increase in intracellular cAMP through stimulation of adenylyl cyclase. Since cAMP can pass through gap junction channels (Bevans *et al.*, 1998), it is possible that propagation of the FSH-generated signal transduction cascade throughout the granulosa cell population depends on gap junctions. However, this alone cannot explain the arrest of folliculogenesis in *Gja1*$^{-}$*/Gja1*$^{-}$ ovaries because follicular development in female mice lacking FSH receptors proceeds to an advanced secondary follicle stage, well beyond the point at which *Gja1*$^{-}$*/Gja1*$^{-}$ follicles arrest (Dierich *et al.*, 1998).

Intraovarian paracrine signaling has also been implicated in early stages of folliculogenesis (reviewed by Eppig *et al.*, 1996; Udoff and Adashi, 1999). One identified paracrine factor is GDF-9 (growth/differentiation factor-9), a TGFβ family member that, within the mouse ovary, is expressed exclusively in growing oocytes from the primary follicle

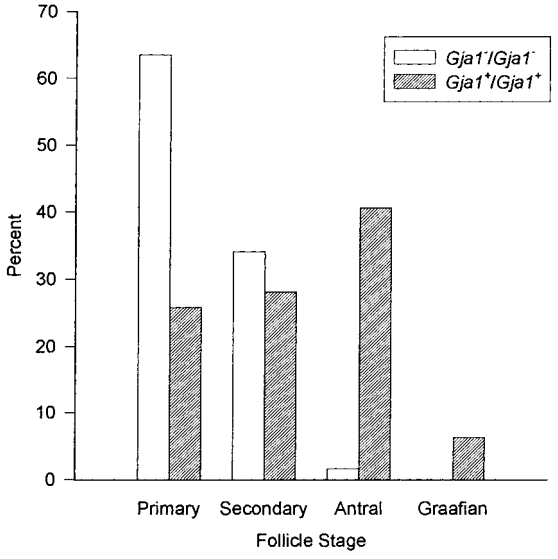

FIG. 7. In contrast to what was observed with the C57BL/6 strain, a range of follicle types can be found in grafted CD1 ovaries lacking Cx43. The distribution of follicle stages reached in CD1 null mutant ovaries was compared with that of wild-type ovaries after 3 weeks of development in kidney grafts. Most of the mutant follicles did not progress beyond the primary follicle stage, but some reached the secondary follicle stage and one antral follicle was observed. $N = 63$ follicles categorized for each genotype.

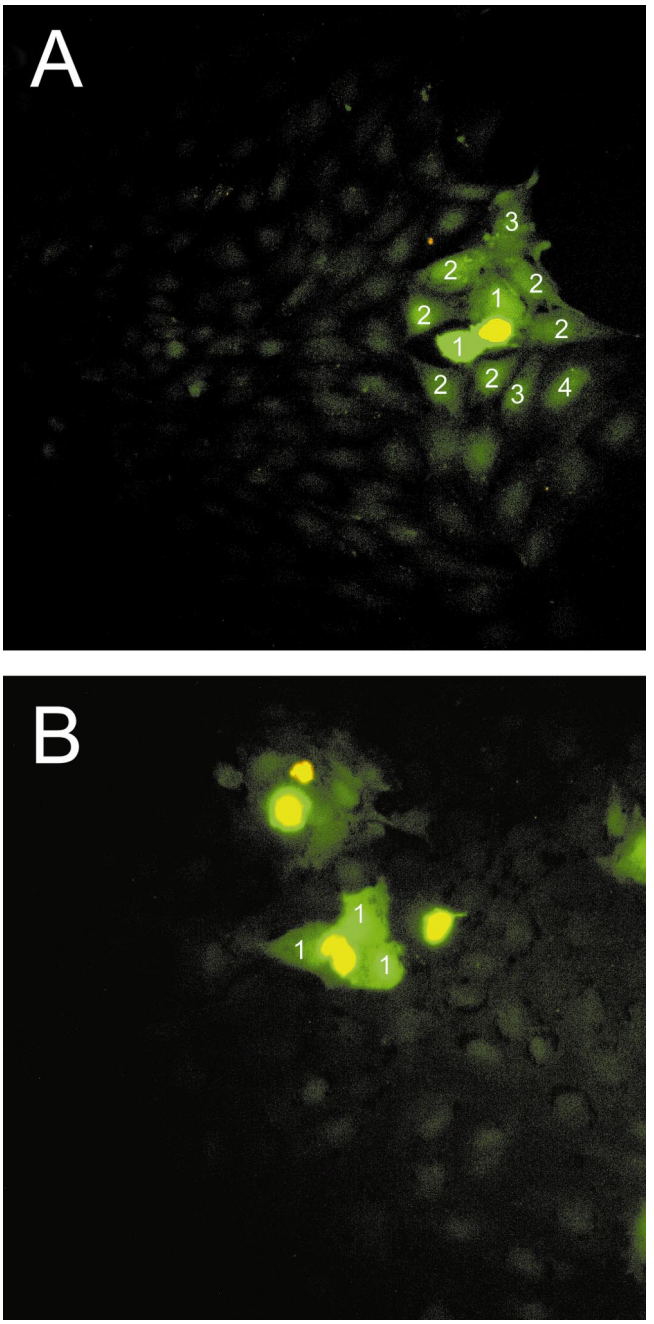

FIG. 8. Gap-junctional coupling between granulosa cells is reduced, but not eliminated, in the absence of Cx43. Wild-type granulosa cells were first loaded with dil and calcein-AM and then seeded onto unlabeled wild-type (A) or null mutant (B) granulosa cells (C57BL/6 strain) from grafted follicles to allow for calcein transfer via gap junctions. Numbers indicate ordinals (first order, second order, etc.) of recipient cells.

stage onward (McGrath *et al.,* 1995; Fitzpatrick *et al.,* 1998; Elvin *et al.,* 1999a). *In vitro* studies have shown that GDF-9 promotes both proliferation and differentiation of granulosa cells (Hayashi *et al.,* 1999; Elvin *et al.,* 1999a). GDF-9-deficient female mice are viable but sterile: folliculogenesis arrests in the primary follicle stage (Dong *et al.,* 1996). GDF-9-deficient oocytes grow to normal size, but at an accelerated rate that is correlated with increased expression of another paracrine signaling molecule, MGF (mast cell growth factor), by the granulosa cells (Elvin *et al.,* 1999b). *Gdf9*$^{-}$/*Gdf9*$^{-}$ oocytes are morphologically abnormal, exhibit reduced meiotic competence, and eventually degenerate (Dong *et al.,* 1996; Carabatsos *et al.,* 1998). The fact that granulosa cell proliferation beyond the unilaminar stage is blocked in both Cx43-deficient and GDF-9-deficient ovaries suggests there may be an interaction between gap-junctional and paracrine signaling pathways during folliculogenesis. However, preliminary results using semiquantitative RT-PCR indicate that GDF-9 expression is maintained in Cx43-deficient ovaries, taking into account the reduced number of oocytes (K. J. Barr and G. M. Kidder, unpublished data). Instead, we hypothesize that the ability of the granulosa cells of primary follicles to maintain their response to GDF-9 depends on their being coupled with one another via Cx43-containing gap junction channels. The receptor for GDF-9 has not been identified but, by analogy with other TGFβ family members, GDF-9 is likely to act through a plasma membrane complex of type I and type II serine/threonine kinase receptors. Ligand binding to the receptor complex results in the activation of SMAD proteins which in turn translocate to the nucleus to bind specific promoters to regulate transcription (reviewed by Derynck *et al.,* 1998). We are also exploring the possibility that GDF-9 signaling is required for maintaining the high level of Cx43 expression and intercellular coupling seen in granulosa cells of developing follicles.

The failure of Cx43-deficient granulosa cells to increase in number beyond the primary or early secondary follicle stage was correlated with morphological defects in the oocytes as well as their failure to acquire meiotic and fertilization competence. Furthermore, growth of the mutant oocytes was retarded. The fact that oocyte development is impaired in follicles in which Cx43 is missing from the granulosa cells underscores the dependency of oocytes on the granulosa cell layers during oogenesis. There is ample experimental evidence that these two cell types influence each other, although in most cases this influence has been ascribed to paracrine factors (reviewed by Eppig, 1994). There are two clear examples of granulosa cells influencing oocyte development via a gap-junctional communication pathway. One is in the regulation of oocyte meiosis: signals which maintain the fully grown oocyte in meiotic arrest despite acquisition of meiotic competence, as well as signals which trigger the resumption of meiosis in response to LH, pass through the oocyte–granulosa cell gap junctions (Coskun and Lin, 1994; Granot and Dekel, 1994; Downs, 1995; Byskov *et al.,* 1997). The second is metabolic coupling: the passage of metabolites from cumulus granulosa cells to their enclosed oocytes. Studies of the uptake of a variety of radiolabeled molecules by denuded or cumulus-enclosed mouse oocytes *in vitro* have demonstrated that such molecules are taken up and metabolized by granulosa cells followed by transfer of the metabolites to the oocyte via gap junctions (Heller *et al.,* 1981). Furthermore, growth of oocytes cultured *in vitro* requires gap-junctional coupling with granulosa cells (Eppig, 1994). This is thought to reflect a requirement for metabolic coupling to support oocyte growth since the rate of transfer of metabolites from granulosa cells to the oocyte and the rate of oocyte growth *in vitro* are directly proportional to the number of granulosa cells attached to the oocyte (Brower and Schultz, 1982). In the present study, the rate of oocyte growth was reduced in mutant ovaries, in which the granulosa cells in most follicles remained as a single layer. The extent of reduction was not great, however, suggesting that the number of granulosa cells attached to the oocyte is not a major determinant of the rate of oocyte growth *in vivo.* On the other hand, it appears to be a very important determinant of oocyte quality.

Our dye transfer assays indicated that the loss of Cx43 results in a reduction, but not the loss, of gap-junctional coupling between granulosa cells. This finding is consistent with the presence of additional connexins in granulosa cells of primary follicles. Although both Cx32 and Cx45 have been detected in granulosa cells of mature follicles (Valdimarsson *et al.,* 1993; Li and Mather, 1997; Okuma *et al.,* 1996; Alcoléa *et al.,* 1999), recent evidence indicates that these connexins do not contribute to granulosa cell gap junctions in preantral follicles (Wright *et al.,* 2000). Thus another connexin, perhaps Cx57, must provide the residual coupling seen in Cx43-deficient granulosa cells (Itahana *et al.,* 1998). In any case, the existence of residual coupling leaves unexplained the severity of the defects in oocyte and follicle development seen in the mutant ovaries. While it is clear that the mutant granulosa cells remain communication competent, it is possible that the preloading assay used here gives an inflated estimate of their true level of intercellular coupling because of the necessity to use wild-type cells as dye donors, which could increase the efficiency with which connexons in the mutant cells are recruited to form intercellular channels. On the other hand, it may be that a substantial level of intercellular coupling is maintained in the mutant granulosa cells *in vivo,* but that Cx43-containing channels play a role in granulosa cell function that other coexpressed connexins are unable to play. It has become clear that gap junction channels composed of different connexins can have different permeabilities, both to artificial tracer molecules and to endogenous metabolites (Bruzzone *et al.,* 1996a,b; Bevans *et al.,* 1998; Goldberg *et al.,* 1999). For example, the permeability of Cx43 channels to ADP/ATP exceeds that of Cx32 channels by more than 100-fold (Goldberg *et al.,* 1999). It remains to be determined whether molecules being transferred between granulosa cells via gap junctions require the unique

properties of Cx43 channels, what those molecules are, and which of them are critical for granulosa cell function.

Connexin knockout mice have begun to provide new insights into the role of GJIC in ovarian development and oogenesis. Disruption of the gene (*Gjb1*) encoding Cx32 resulted in females that are both viable and fertile (Nelles *et al.*, 1996). This indicates that Cx32 in the gap junctions joining granulosa cells is dispensable, presumably because the functions served by Cx32 channels in this context, unlike those served by Cx43 channels, are not unique. The gene encoding Cx45 (*Gja7*) has also been knocked out but the homozygous mutant offspring die in midgestation, too early to investigate effects on ovarian function (Krüger *et al.*, 2000; Kumai *et al.*, 2000). Mice lacking Cx37, encoded by the *Gja4* gene, are viable and ovarian folliculogenesis proceeds in apparently normal fashion until the tertiary (antral) follicle stage (Simon *et al.*, 1997). Mature Graafian follicles never develop, however, and ovulation in response to gonadotropin stimulation does not occur. The mutation appears to abolish both gap junctions and intercellular coupling between cumulus granulosa cells and oocytes, the latter failing to achieve meiotic competence. Eventually, the mutant ovaries become filled with structures resembling corpora lutea, as though the granulosa cells have differentiated prematurely as luteal cells. It therefore appears that GJIC between oocytes and granulosa cells via Cx37 channels is required to maintain the differentiated state of the granulosa cells, preventing them from luteinizing before ovulation. The very different ovarian phenotypes resulting from the *Gja4* (Cx37) and *Gja1* (Cx43) knockouts indicate that gap-junctional coupling between the oocyte and its cumulus granulosa cells serves a role different from that between the granulosa cells themselves, possibly involving different permeant molecules. Thus, while the follicle is a functional syncytium coupled by gap junctions, it is probably incorrect to suppose that the same molecules pass freely throughout.

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